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TETANUS TOXIN POLYPEPTIDES

Field of the invention

This invention relates to polypeptides derived from fragment C of the tetanus toxin and their use in vaccine compositions.

Background to the invention

Tetanus is a highly infectious, worldwide disease caused by the organism *Clostridium tetani*. Its spores can remain dormant for indefinite periods in house dust and soil which are the prime sources of infection for open wounds. Contaminated animal faeces are also responsible for the spread of the disease and may further contaminate previously uninfected locales.

Tetanus infection is always a risk for the wounded and is usually associated with great pain and may even lead to death. Thus, in the nineteenth century, when the causative agent was discovered to be a bacterium, great effort was devoted to the development of an effective vaccine. The first successful vaccination in human subjects was carried out in 1926, when the vaccine used comprised crude tetanus toxin treated to render it atoxic.

Tetanus toxin provided the target for the first vaccine as it had already been discovered that symptoms are not due to the bacterium infecting host cells but rather by a potent neurotoxin released upon lysis of the bacterial cells.

In vivo, a single (parent) peptide is synthesised which comprises both light and heavy chains. On lysis of the bacterial cell, an endogenous protein cleaves the peptide between a preexisting disulphide bridge to give two peptide chains linked by a disulphide bridge.

Papain cleaves the heavy chain to give a fragment B (100 kDa) consisting of the light chain and part of the heavy chain, and a fragment C (50 kDa). consisting of the remainder of the heavy chain. It has been shown that both fragments are capable of providing protection but

may be associated with residual toxicity that would be due to contamination with uncleaved toxin.

Tetanus toxin (TeNT) is a member of the family of clostridial neurotoxins (CNTs) which includes the botulinum toxins (BoNTs). CNTs inhibit neurotransmitter release from presynaptic neuronal cells by proteolytic cleavage of proteins involved in the fusion of synaptic vesicles with the cell membrane. This cleavage is catalysed by the 50 kDa L-chain of CNTs which encodes a zinc dependent metaloprotease. The overall structural and functional properties of the CNTs are very similar, having a similar sub-unit structure and 30-40% amino acid identity (Minton, 1995). In contrast to BoNTs which remain within the peripheral nervous system, TeNT is trafficked to the central nervous system by retrograde axonal transport followed by trans-synaptic spread into inhibitory interneurons. It is this differential cellular trafficking of TeNT compared to the BoNTs which explains the distinct sites of action of the toxins and therefore the distinct clinical symptoms of the two diseases.

The mechanism whereby TeNT and BoNT bind to sensitive cells has been studied in a variety of *in vitro* systems including tissue preparations, primary cell cultures and cell lines. These studies, and others, have shown that TeNT and BoNT bind to membrane receptors, and are internalised prior to the cytosolic action of the L-chain of the toxins. The steps involved in binding and trafficking of TeNT are largely uncharacterised, although it appears to involve internalisation through uptake of small synaptic vesicles during vesicle reuptake. The binding of TeNT to neuronal tissue involves the C-terminal 50 kDa domain (H_C domain, also known as fragment C), as demonstrated by retrograde transport of H_C *in vivo* and its ability to bind rat brain membranes and primary neuronal cells and neuroblastoma cell lines *in vitro*. When added to spinal cord cells, TeNT H_C fragment blocked uptake of TeNT as shown by inhibition of VAMP-2 cleavage and antagonism of neuromuscular transmission, indicating that the binding of H_C is functional. The H_C fragment alone retains the ability to undergo retrograde transport *in vivo*, and has been used as a carrier of foreign proteins to the CNS.

Since the initial demonstration of TeNT binding to gangliosides, many studies have attempted to characterise binding of CNTs to gangliosides using *in vitro* and *in vivo* assays. It is well documented that both TeNT and the isolated H_C fragment bind gangliosides GD1b and GT1b but not GM1, demonstrating the requirement for poly-sialic acids within the ganglioside for binding. The requirement for gangliosides on cell surfaces was shown by incubation of cultured chromaffin cells with GD1b or GT1b which increased their sensitivity to TeNT and BoNT. Recent experiments involving treatment of primary spinal cord neurons grown in the presence of fumonisin, an inhibitor of ganglioside synthesis, has shown that fumonisin treated cells became insensitive to TeNT, as demonstrated by lack of blockade of potassium - induced stimulation of glycine release and retention of VAMP-reactivity. Furthermore, fumonisin treated cells regained sensitivity to TeNT upon addition of exogenous gangliosides. However, much experimental evidence is inconsistent with gangliosides being the sole receptor for TeNT and BoNTs. For example, both BoNTs and TeNT both bind gangliosides but have different sites of action within the body, polysialogangliosides are not present uniquely on neuronal tissue, and TeNT binding to rat brain membranes and neuronal cell lines is protease sensitive. Thus a dual receptor model, invoking toxin interacting with both gangliosides and protein receptors has been proposed. In support of this model, synaptotagmin II, which is localised in the lumen of small synaptic vesicles, has been identified as a protein receptor for BoNT serotype B, and serotypes A and E. Cross-linking experiments using primary spinal cord motoneurons and NGF-differentiated PC12 cells have identified a 15 kDa glycoprotein as a possible neuronal receptor for TeNT (Herreros, et al. J. Neurochemsitry in press). This receptor binds only to the carboxy terminal half of the H_C domain of TeNT.

Existing licensed vaccines are based on the inactivated toxin obtained from lysed *C. tetani* cells. To obtain sufficient toxin requires large cell-cultures which tends to give rise to toxin being contaminated with cell debris. The resulting toxin is rendered atoxic (toxoided) by treatment, usually with formaldehyde, but may also be toxoided with glutaraldehyde. However, while rendering the toxin harmless, either treatment may also lead to conjugation of the toxin with cell contaminants, which in turn may lead to possible adverse clinical reactions.

The structural gene for tetanus toxin has been cloned and sequenced (Fairweather *et al.*, 1986) and this has enabled the production of recombinant TeNT. Recombinant vaccines based on the fragment C of TeNT have been described in the art (see for example WO-A-9015871 and EP-A-209281). However, such vaccines retain ganglioside binding activity. Although there is no conclusive evidence that ganglioside binding is associated with toxicity in immunised individuals, it would be desirable to obtain a TeNT polypeptide with reduced ganglioside binding activity.

Studies of the ganglioside binding properties of the H_C fragment employing deletion mutants expressed in *E.coli* have shown that some mutants lacking the N-terminal region of H_C could still bind gangliosides and neuronal cells, but those lacking carboxy terminal amino acids were defective in binding. The studies by Halpern et al (Halpern and Loftus, 1993) suggested an essential role for the carboxy terminal 10 amino acids (residues 1306-1315) of H_C in both ganglioside and neuronal cell binding. Studies in which H_C was bound to a GT1b ganglioside photoaffinity ligand revealed modification of His-1293 within the carboxy-terminal 34 amino acid peptide of H_C (1282 – 1315) (Shapiro *et al.*, 1997). This result, taken together with inhibition studies using unlabelled GT1b ganglioside, suggested the 34 amino acid peptide of H_C may be sufficient for binding to gangliosides GT1b.

Mutant molecules were produced in *E.coli*, purified and assayed in ganglioside binding, cell binding and *in vivo* retrograde transport assays. Our results identify specific residues that are required for ganglioside binding, and identify certain mutants with reduced ganglioside binding but which still retain the ability to bind primary neuronal cells and undergo retrograde axonal transport.

Summary of the Invention

We have constructed a series of site-directed mutants of TeNT, using the structural information provided by the three dimensional structure reported recently (Umland *et al.*, 1997). Mutant molecules were produced in *E.coli*, purified and assayed in ganglioside binding, cell binding and *in vivo* retrograde transport assays. Our results confirm a role for His 1293 in ganglioside binding. However, we have also now identified other regions of

the TeNT fragment C required for binding gangliosides, deletion of which leads to a greater reduction in ganglioside binding than the results obtained for His 1293 alone. Typically, the regions are loop regions which connect two sections of β sheet. Our results also identify certain mutants with reduced ganglioside binding but which still retain the ability to bind primary neuronal cells and undergo retrograde axonal transport.

Accordingly, the present invention provides a polypeptide comprising tetanus toxin (TeNT) fragment C, or an immunogenic fragment thereof, which tetanus toxin fragment C, or immunogenic fragment thereof comprises a mutation in a loop region, which mutation results in a reduction in the binding of the tetanus toxin fragment C, or immunogenic fragment thereof, to gangliosides.

The present invention also provides a polypeptide comprising tetanus toxin (TeNT) fragment C, or an immunogenic fragment thereof, which tetanus toxin fragment C, or immunogenic fragment thereof comprises a mutation in a loop region, which mutation results in a reduction in the binding of the tetanus toxin fragment C, or immunogenic fragment thereof, to primary motoneurones.

The present invention also provides a polypeptide comprising tetanus toxin (TeNT) fragment C, or an immunogenic fragment thereof, which tetanus toxin fragment C, or immunogenic fragment thereof comprises a mutation in a loop region, which mutation results in a reduction in the ability of the tetanus toxin fragment C, or immunogenic fragment thereof, to undergo retrograde transport.

Preferably the mutation results in a reduction in the binding of the tetanus toxin fragment C (or immunogenic fragment thereof) to gangliosides *and* a reduction in the binding to primary motoneurones and/or a reduction in the ability to undergo retrograde transport.

Preferably said loop region is present in the full length wild type sequence between two β sheets. More preferably said loop region is selected from amino acid residues 1214 to 1219 and 1271 to 1282 of the amino acid sequence of TeNT fragment C.

Preferably said mutation is at least one deletion, more preferably at least one deletion selected from Δ1214 to 1219, Δ1274 to 1279 and Δ1271 to 1282 of the amino acid sequence of TeNT fragment C.

The present invention further provides a polynucleotide encoding a polypeptide of the invention.

The present invention also provides vectors comprising a polynucleotide encoding a polypeptide of the invention operably linked to a regulatory sequence. Preferably the regulatory sequence allows expression of the polypeptide in a host cell. Typically the host cell is a bacterium, or a cell of an animal, more preferably a mammal, including primates and humans.

The polypeptides, polynucleotides and vectors of the present invention may be used in the prevention (or reduction in susceptibility to), or treatment of, *Clostridium tetani* infections. Thus, in a further aspect, the present invention provides a pharmaceutical composition comprising a polypeptide, polynucleotide or vector of the invention together with a pharmaceutically acceptable carrier or diluent.

The present invention also provides a method of treating or preventing *C. tetani* infection in a human or animal which comprises administering to the human or animal an effective amount of a polypeptide, polynucleotide or vector of the invention.

The present invention further provides a polypeptide of the present invention for use in therapy, such as the prevention (or reduction in susceptibility to), or treatment of, *C. tetani* infections. Also provided is the use of a polypeptide of the invention in the manufacture of a medicament for use in the prevention (or reduction in susceptibility to), or treatment of, *C. tetani* infections.

In another aspect, the invention provides the use of a polypeptide, polynucleotide or vector of the invention in a method for producing antibodies which recognise epitopes within a TeNT polypeptide. For example, the present invention provides a method for producing

antibodies which recognise epitopes within a TeNT polypeptide which method comprises administering a polypeptide, a polynucleotide or a vector of the invention to a mammal.. Such antibodies produced by the various methods known in the art may be used in a method of treating *C. tetani* infection in a human or animal which comprises administering to a human or animal an effective amount of an antibody produced by the above method of the invention.

In a further aspect, the invention provides a method for reducing the binding affinity of a TeNT fragment C polypeptide for gangliosides which method comprises modifying one or more amino acid residues present in a surface-exposed loop region of the polypeptide. Also provided is a polypeptide produced by said method.

Detailed Description of the Invention

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc.

A. TeNT Polypeptides

The structural gene for tetanus toxin has been cloned and sequenced (Fairweather *et al.*, 1986; Eisel *et al.*, 1986 (also database accession No. BTCLTN and g69647)). The amino acid sequence is provided herein for reference. Fragment C is a 50 kDa polypeptide generated by papain cleavage and comprises or substantially corresponds to the 451 amino acids at the C-terminus. The terms "fragment C" and "H_C" are used interchangeably herein. It is to be understood that although the description below adopts the amino acid numbering based on the sequence of tetanus toxin described herein, the present invention is equally applicable to fragment C variants found in other strains of *C. tetani* (which may have slightly different amino acid numbering).

Polypeptides of the invention comprise tetanus toxin fragment C or immunogenic

fragments thereof that have been modified to reduce their ability to bind to gangliosides, and/or their ability to bind primary motoneurones, and/or their ability to undergo retrograde transport. In particular, the polypeptides of the invention comprise mutations in a loop region found between 2 β sheets. The 3D structure of TeNT fragment C has been determined by Umland *et al.* (1997) the structural coordinates deposited in the Protein Data Bank under reference number 1af9. The structural information presented in Umland *et al.* (1997) (see Figure 1a in particular) allows the skilled person to identify candidate loop regions. Preferably these loops are exposed on the surface of the molecule. These may then be mutagenised and tested for ganglioside binding using, for example, the binding assays described in the Examples. Two such loops which have been shown to be involved in ganglioside binding are described in the Examples. The first loop consists of approximately amino acids 1214 to 1219 and the second loop consists of approximately amino acids 1271 to 1282.

Mutations in the loop regions may be substitutions, insertions and/or deletions. Preferably the mutations are deletions, more preferably deletions which remove substantially all of a particular loop region. Techniques for modifying amino acids sequences are well known in the art, such as PCR-directed mutagenesis.

It is preferred that mutant TeNT polypeptides of the present invention have less than 50% ganglioside binding activity compared with wild type TeNT, more preferably less than 40, 30, 20 or 10%, most preferably less than 5%. Ganglioside binding activity may, for example, be determined *in vitro* as described in the examples.

Alternatively, or preferably in addition, it is preferred that mutant TeNT polypeptides of the present invention have less than 50% neuronal cell binding activity compared with wild type TeNT, more preferably less than 40, 30, 20 or 10%, most preferably less than 5%. Neuronal cell binding activity may, for example, be determined as described in the examples.

In addition, it is preferred that mutant polypeptides of the invention retain at least 50% of the immunogenicity of the wild type sequence from which they are derived (i.e. the full

length fragment C sequence or immunogenic fragments thereof), more preferably at least 70, 80 or 90%. Immunogenicity may be determined typically by the use of *in vitro* techniques such as ELISA or Western blotting. Alternatively, or in addition, immunogenicity may be determined *in vivo* by, for example, immunising animals with a polypeptide of the invention and then either testing sera by ELISA or Western blotting, or by subsequently challenging immunised individuals with active toxin.

In a particularly preferred embodiment, a polypeptide of the invention comprises TeNT fragment C, or an immunogenic fragment thereof, which TeNT fragment C, or immunogenic fragment thereof, comprise a mutation in amino acid residues 1214 to 1219 and/or 1271 to 1282. In particular a polypeptide of the invention comprises TeNT fragment C, or an immunogenic fragment thereof, which TeNT fragment C, or immunogenic fragment thereof, comprises a deletion of amino acid residues 1214 to 1219 and/or at least 1274 to 1279, more preferably 1271 to 1282.

In another preferred embodiment, the polypeptides of the invention further comprise a modification at residue His-1293, preferably a substitution such as His→Ala or His→ Ser.

Fragments of tetanus toxin fragment C that contain epitopes may also be used in the polypeptides of the invention. These fragments will comprise at least 5 or 6 amino acids, preferably at least 10 amino acids, more preferably at least 15, 20, 50 or 100 amino acids. Particularly preferred fragments include from about amino acids 943 to 1023, which is a good B-cell epitope, and from about amino acids 946 to 966 which is a good T-cell epitope. All amino acid numbering is with reference to the full-length toxin gene.

The amino acid sequence of tetanus toxin fragment C can be further modified to provide polypeptides of the invention. For example, this may be carried out to enhance the immunogenicity of the polypeptides of the invention. Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions provided that the modified polypeptide retains epitopes.

Conservative substitutions may be made, for example according to the Table below.

Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

Polypeptides of the invention may further comprise heterologous amino acid sequences, typically at the N-terminus or C-terminus, preferably the N-terminus. Heterologous sequences may include sequences that affect intra or extracellular protein targeting (such as leader sequences). Heterologous sequences may also include sequences that increase the immunogenicity of the polypeptide of the invention and/or which facilitate identification, extraction and/or purification of the polypeptides. Another heterologous sequence that is particularly preferred is a polyamino acid sequence such as polyhistidine which is preferably N-terminal. A polyhistidine sequence of at least 10 amino acids, preferably at least 17 amino acids but fewer than 50 amino acids is especially preferred.

Other heterologous amino acid sequences includes immunogenic sequences from other pathogenic organisms such as bacteria or viruses. Examples include pathogenic *E. coli, Neiserria sp., B. pertussis, C. difficile, Salmonella sp., Campylobacter sp., P. falciparum*, hepatitis B virus, hepatitis C virus and human papilloma virus. Conveniently, these immunogenic sequences may be inserted into a loop region which it is desired to disrupt (such as amino acids 1214 to 1219).

Polypeptides of the invention are typically made by recombinant means, for example as described below. However they may also be made by synthetic means using techniques well known to skilled persons such as solid phase synthesis. Polypeptides of the invention may also be produced as fusion proteins, for example to aid in extraction and purification.

Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and β-galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences, such as a thrombin cleavage site. Preferably the fusion protein will not hinder the function of the protein of interest sequence.

Polypeptides of the invention may be in a substantially isolated form. It will be understood that the protein may be mixed with carriers or diluents which will not interfere with the intended purpose of the protein and still be regarded as substantially isolated. A polypeptide of the invention may also be in a substantially purified form, in which case it will generally comprise the protein in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the protein in the preparation is a polypeptide of the invention.

B. Polynucleotides and vectors.

Polynucleotides of the invention comprise nucleic acid sequences encoding the polypeptides of the invention. Polynucleotides of the invention may comprise DNA or RNA. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides of the invention.

Preferred polynucleotides of the invention also include polynucleotides encoding any of the polypeptides of the invention described above. It will be understood by a skilled person that numerous different polynucleotides can encode the same polypeptide as a result of the degeneracy of the genetic code.

Polynucleotides of the invention can be incorporated into a recombinant replicable vector.

The vector may be used to replicate the nucleic acid in a compatible host cell. Thus, in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *E. coli*, yeast, mammalian cell lines and other eukaryotic cell lines, for example insect Sf9 cells.

Preferably, a polynucleotide of the invention in a vector is operably linked to a regulatory sequence that is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

Such vectors may be transformed or transfected into a suitable host cell as described above to provide for expression of a polypeptide of the invention. This process may comprise culturing a host cell transformed with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the polypeptides, and optionally recovering the expressed polypeptides.

The vectors may be, for example, plasmid or virus vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell. The vector may also be adapted to be used *in vivo*, for example in a method of gene therapy.

Promoters/enhancers and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. For example,

prokaryotic promoters may be used, in particular those suitable for use in *E. coli* strains (such as *E. coli* HB101). In a particularly preferred embodiment of the invention, an htrA or nirB promoter may be used. When expression of the polypeptides of the invention is carried out in mammalian cells, either *in vitro* or *in vivo*, mammalian promoters may be used. Tissue-specific promoters may also be used. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the promoter rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, herpes simplex virus promoters or adenovirus promoters. All these promoters are readily available in the art.

C. Host cells

Vectors and polynucleotides of the invention may be introduced into host cells for the purpose of replicating the vectors/polynucleotides and/or expressing the polypeptides of the invention encoded by the polynucleotides of the invention. Suitable host cells include prokaryotes such as eubacteria, for example *E. coli* and *B. subtilis* and eukaryotes such as yeast, insect or mammalian cells.

Vectors/polynucleotides of the invention may be introduced into suitable host cells using a variety of techniques known in the art, such as transfection, transformation and electroporation. Where vectors/polynucleotides of the invention are to be administered to animals, several techniques are known in the art, for example infection with recombinant viral vectors such as retroviruses, herpes simplex viruses and adenoviruses, direct injection of nucleic acids and biolistic transformation.

D. Protein Expression and Purification

Host cells comprising polynucleotides of the invention may be used to express polypeptides of the invention. Host cells may be cultured under suitable conditions which allow expression of the proteins of the invention. Expression of the polypeptides of the invention may be constitutive such that they are continually produced, or inducible, requiring a stimulus to initiate expression. In the case of inducible expression, protein production can be initiated when required by, for example, addition of an inducer

substance to the culture medium, for example dexamethasone or IPTG. Suitable methods for producing tetanus toxin fragment C polypeptides are described in WO-A-9015871 and EP-A-209281.

Polypeptides of the invention can be extracted from host cells by a variety of techniques known in the art, including enzymatic, chemical and/or osmotic lysis and physical disruption.

Purification of polypeptides may optionally be performed using well known techniques such as affinity chromatography, including immunoaffinity chromatography, ion-exchange chromatography and the like. A particularly preferred technique is to express the polypeptide of the invention as a fusion protein with polyhistidine tag (for example 6xHis) and purify cell extracts using Ni-NTA agarose (Qiagen). A variety of other similar affinity chromatography systems based on fusion protein sequences are known in the art.

Polypeptides of the invention may also be produced recombinantly in an *in vitro* cell-free system, such as the TnT™ (Promega) rabbit reticulocyte system.

E. Administration

The polypeptides of the invention may be administered by direct injection. Preferably the polypeptides are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition, which may be for human or veterinary use. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration. Typically, each polypeptide is administered at a dose of from 0.01 to 30 μg/kg body weight, preferably from 0.1 to 10 μg/kg, more preferably from 0.1 to 1 μg/kg body weight. It is also possible to use antibodies prepared using the polypeptides of the invention, as described below, in treating or preventing *C. tetani* infection. Neutralising antibodies, or fragments thereof which retain specificity for *C. tetani* antigens, can be administered in a similar manner to the polypeptides of the invention.

The polynucleotides of the invention may be administered directly as a naked nucleic acid construct, preferably further comprising flanking sequences homologous to the host cell genome. When the expression cassette is administered as a naked nucleic acid, the amount of nucleic acid administered is typically in the range of from 1 μg to 10 mg, preferably from 100 μg to 1 mg.

Uptake of naked nucleic acid constructs by mammalian cells is enhanced by several known transfection techniques for example those including the use of transfection agents. Example of these agents include cationic agents (for example calcium phosphate and DEAE-dextran) and lipofectants (for example lipofectam™ and transfectam™). Typically, nucleic acid constructs are mixed with the transfection agent to produce a composition.

In a particularly preferred embodiment, a nucleotide of the invention is introduced into an attenuated strain of *Salmonella sp.* such that a polypeptide of the invention is expressed by the transformed bacterial strain and the live bacteria administered, typically orally, to a patient.

Preferably the polynucleotide or vector of the invention is combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

F. Preparation of Vaccines

Vaccines may be prepared from one or more polypeptides of the invention. The preparation

of vaccines which contain an immunogenic polypeptide(s) as active ingredient(s), is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof.

In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. Examples of adjuvants which may be effective include but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion.

Further examples of adjuvants and other agents include aluminum hydroxide, aluminum phosphate, aluminum potassium sulfate (alum), beryllium sulfate, silica, kaolin, carbon, water-in-oil emulsions, oil-in-water emulsions, muramyl dipeptide, bacterial endotoxin, lipid X, *Corynebacterium parvum* (*Propionobacterium acnes*), *Bordetella pertussis*, polyribonucleotides, sodium alginate, lanolin, lysolecithin, vitamin A, saponin, liposomes, levamisole, DEAE-dextran, blocked copolymers or other synthetic adjuvants. Such adjuvants are available commercially from various sources, for example, Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.) or Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, Michigan).

Typically, adjuvants such as Amphigen (oil-in-water), Alhydrogel (aluminum hydroxide), or a mixture of Amphigen and Alhydrogel are used. Only aluminum hydroxide is approved

for human use.

The proportion of immunogen and adjuvant can be varied over a broad range so long as both are present in effective amounts. For example, aluminum hydroxide can be present in an amount of about 0.5% of the vaccine mixture (Al_2O_3 basis). Conveniently, the vaccines are formulated to contain a final concentration of immunogen in the range of from 0.2 to 200 μg/ml, preferably 5 to 50 μg/ml, most preferably 15 μg/ml.

After formulation, the vaccine may be incorporated into a sterile container which is then sealed and stored at a low temperature, for example 4°C, or it may be freeze-dried. Lyophilisation permits long-term storage in a stabilised form.

The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against an immunogenic polypeptide containing a TeNT antigenic sequence resulting from administration of this polypeptide in vaccines which are also comprised of the various adjuvants.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1% to 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10% to 95% of active ingredient, preferably 25% to 70%. Where the vaccine composition is lyophilised, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is preferably effected in buffer

Capsules, tablets and pills for oral administration to a patient may be provided with an

enteric coating comprising, for example, Eudragit "S", Eudragit "L", cellulose acetate, cellulose acetate phthalate or hydroxypropylmethyl cellulose.

The polypeptides of the invention may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric and maleic. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine and procaine.

G. Dosage and Administration of Vaccines

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. The quantity to be administered, which is generally in the range of 5 μg to 250 μg of antigen per dose, depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and the degree of protection desired. A preferable range is from about 20 μg to about 40 μg per dose.

A suitable dose size is about 0.5 ml. Accordingly, a dose for intramuscular injection, for example, would comprise 0.5 ml containing 20 μg of immunogen in admixture with 0.5% aluminum hydroxide.

Precise amounts of active ingredient required to be administered may depend on the judgement of the practitioner and may be peculiar to each subject.

The vaccine may be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1 to 4 months

for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the need of the individual and be dependent upon the judgement of the practitioner.

In addition, the vaccine containing the immunogenic TeNT antigen(s) may be administered in conjunction with other immunoregulatory agents, for example, immunoglobulins.

H. Preparation of antibodies against the polypeptides of the invention

The immunogenic polypeptides prepared as described above can be used to produce antibodies, both polyclonal and monoclonal. If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with an immunogenic polypeptide bearing a TeNT epitope(s). Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to a TeNT epitope contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art.

Monoclonal antibodies directed against TeNT epitopes in the polypeptides of the invention can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced against TeNT epitopes can be screened for various properties; i.e., for isotype and epitope affinity.

An alternative technique involves screening phage display libraries where, for example the phage express scFv fragments on the surface of their coat with a large variety of complementarity determining regions (CDRs). This technique is well known in the art.

Antibodies, both monoclonal and polyclonal, which are directed against TeNT epitopes are particularly useful in diagnosis, and those which are neutralising are useful in passive

immunotherapy. Monoclonal antibodies, in particular, may be used to raise anti-idiotype antibodies. Anti-idiotype antibodies are immunoglobulins which carry an "internal image" of the antigen of the infectious agent against which protection is desired.

Techniques for raising anti-idiotype antibodies are known in the art. These anti-idiotype antibodies may also be useful for treatment of *C. tetani*, as well as for an elucidation of the immunogenic regions of *C. tetani* antigens.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes fragments of whole antibodies which retain their binding activity for a target antigen. Such fragments include Fv, F(ab') and $F(ab')_2$ fragments, as well as single chain antibodies (scFv). Furthermore, the antibodies and fragments thereof may be humanised antibodies, for example as described in EP-A-239400.

The invention will be described with reference to the following Examples which are intended to be illustrative only and not limiting. The Examples refer to the Figures. Referring to the Figures in more details:

Description of the Figures

Figure 1 is a 3D representation of the structure of tetanus toxin fragment C.

Figure 2 is a graph showing % binding to gangliosides compared with wild type for various TeNT fragment C mutants.

EXAMPLES

METHODS

Bacterial strains and plasmid construction

E. coli strain BL21 (*λDE3, ompT, hsdS$_B$ (r_B-m_B-), gal, dcm*) was used as the host for the plasmids described below. Plasmid pKS1 contains a codon-optimised gene for the expression of the Hc fragment of TeNT under the control of the T7 promoter. It was created by PCR amplification (Pfu polymerase, Stratagene, Cambridge UK) of a 1357 bp fragment using pTETtac215 (Makoff *et al.*, 1989) as template and the oligonucleotides 5'GAGCATATGAAAAACCTTGAT and 5'CGGATCCTTAGTCGTTGGTCCA which introduce *Nde*I and *Bam*HI sites at the 5' and 3' ends of the gene respectively. After blunt end ligation of the PCR product into the vector pCRScript (Stratagene) to form plasmid pJC6, the *Nde*I - *Bam*HI fragment was purified by agarose gel electrophoresis using a Qiaex II gel purification kit (Qiagen, West Sussex, UK), and subloned into pET28a (Novagen, Cambridge UK) which had previously been digested with NdeI and BamHI (Roche Molecular Biochemicals, East Sussex, UK). DNA manipulations were performed by standard procedures.

Mutagenesis and DNA sequencing

Mutagenesis was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene). Pairs of complementary oligonucleotides were used to construct mutant H_C molecules (see Table 1). To create mutants containing single amino acid mutations (M15, M24, M667, M564 and M57), the following cycles were used with pKS1 as template DNA: Step 1: 95°C 30 sec, step 2: 95 °C 30 sec, step 3: 55 °C 1 min, step 4: 68 °C 12 min. Steps 2 to 4 were repeated for 12 cycles.

TABLE 1 Mutants of TeNT H_C constructed

Mutant name	Oligonucleotides used for site directed mutagenesis
M5	NF38: 5' to 3' GGTTGCGACTGGTACTTCTAAGGATCCGAATTCG NF41: 3' to 5'CGAATTCGGATCCTTAGAAGTACCAGTCGCAACC
T1308A	NF49: 5' to 3' GACTGGTACTTCGTTCCGGCTGATGAAGGTTGGA NF50: 3' to 5' GGTCCAACCTTCATCAGCCGGAACGAAGTACCAG
D1309A	NF51: 5' to 3' TGGTACTTCGTTCCGACCGCTGAAGGTTGGACCA NF52: 3' to 5' CGTTGGTCCAACCTTCAGCGGTCGGAACGAAGTA
E1310A	NF57: 5' to 3' TACTTCGTTCCGACCGATGCTGGTTGGACCAACGAC NF58: 3' to 5' GTCGTTGGTCCAACCAGCATCGGTCGGAACGAAGTA
M13	NF47: 5' to 3' TTCGTTCCGACCGATGAATAAGGATCCGAATTCG NF48: 3' to 5' CGAATTCGGATCCTTATTCATCGGTCGGAACGAA
M28	NF79: 5' to 3' GGTACCCACAACGGTCAGCCGAACCGTGACATCCTG NF80: 5' to 3' CAGGATGTCACGGTTCGGCTGACCGTTGTGGGTACC
M37	NF81: 5' to 3' CTGGGTCTGGTTGGTACCAACGACCCGAACCGTGAC NF82: 5' to 3' GTCACGGTTCGGGTCGTTGGTACCAACCAGACCCAG
M40	NF79: 5' to 3' GGTACCCACAACGGTCAGCCGAACCGTGACATCCTG NF80: 5' to 3' CAGGATGTCACGGTTCGGCTGACCGTTGTGGGTACC and NF32: 5' to 3' CTTCTAACTGGTACTTCAACTCTCTGAAAGACAAAATCCTGGG NF33: 3' to 5' CCCAGGATTTTGTCTTTCAGAGAGTTGAAGTACCAGTTAGAAG
M58	NF91: 5' to 3' GTTGGTTACCCGAAACTGCAGAACCTGGACAGAATT NF92: 3' to 5' AATTCTGTCCAGGTTCTGCAGTTTCGGGTAACCAAC
M564	NF32: 5' to 3' CTTCTAACTGGTACTTCAACTCTCTGAAAGACAAAATCCTGGG NF33: 3' to 5' CCCAGGATTTTGTCTTTCAGAGAGTTGAAGTACCAGTTAGAAG
M567	NF97: 5' to 3' CTAACTGGTACTTCAACGCTCTGAAAGACAAAATCCTGGG NF98: 3' to 5' CCCAGGATTTTGTCTTTCAGAGCGTTGAAGTACCAGTTAG

To construct molecules containing deletions (M5, M13, M28 and M58) longer extension times were used with pKS1 as template DNA in the following cycles: Step 1: 95°C 30 sec, step 2: 95 °C 30 sec, step 3: 55 °C 1 min, step 4: 68 °C 18 min. Steps 2 to 4 were repeated for 18 cycles. To construct mutant M37 and the double mutant M40, identical cycling conditions were used, but using M28 mutant DNA as a template.

DNA sequencing

The DNA sequence of the entire insert of pKS1 encoding HC and of all mutants in the regions altered was determined by automated DNA sequencing.

Purification of recombinant His-tagged proteins

250 ml of LB broth containing kanamycin (50 µg/ml) were inoculated with 5 ml of an overnight culture of *E. coli* BL21 cells containing pKS1 or derivatives encoding mutant H_C

genes. At an OD_{650} = 0.8, expression of wild type or mutant H_C protein was induced by the addition of isopropyl-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM. Incubation at 37°C was continued for 3.5 hours, after which cells were recovered by centrifugation at 4000 g for 15 min. Cell pellets were frozen and stored at –20°C. To purify the His-tagged proteins, cell pellets were resuspended in 10 ml of buffer A (300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) containing lysozyme (1 mg/ml) (Roche Molecular Biochemicals) and 10 mM imidazole (Sigma, Dorset, UK).

Cells were sonicated on ice with a flat tip probe for 4.5 minutes in pulses of 15 sec on, 15 sec off using a 550W sonicator (Ultrasonic Processor XL2020, Heat Systems, New York, USA). After centrifugation at 13,000 g for 20 min, 500 µl of Ni-NTA agarose resin (Qiagen) was added and the mixture incubated whilst rotating for 16 h at 4°C. The resin was then centrifuged at 200 g and resuspended in 10 ml buffer A containing 20 mM imidazole. The centriguation and resuspension was repeated twice. The resin was resuspended in 5 ml buffer A and was poured into a disposable plastic column (Biorad, Herts, UK) and the liquid allowed to drain away. The His-tagged proteins were eluted by application of elution buffer (buffer A containing 250 mM imidazole). The protein concentration of the eluted fractions was monitored by measurement at OD_{280}. Peak fractions were pooled and dialysed in buffer A, and protein concentration was measured using a Micro BCA protein assay (Pierce, Chester, UK). Between 20 and 32 mg of purified protein was obtained per litre of bacterial culture.

Biotinylation of Hc protein

Biotinylation of HC protein was carried out using the EZ-Link Sulfo-NHS-LC-biotinylation kit (Pierce).

Ganglioside binding in vitro

(i) Direct binding assay

Direct binding of proteins to gangliosides was measured as follows. ELISA plates (Life Technologies, Paisley, UK) were coated with 10 µg/ml bovine gangliosides $GT1_b$ (Sigma, Dorset, UK) in 50 µl methanol and allowed to dry overnight. After two washes of 3 min in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS/T). 100 µl of blocking

solution (PBS containing 3% BSA (Sigma)) was added, and the plates blocked for 1 hour at 37°C. All subsequent incubations were at 37°C in 50 µl volumes, and all washes were for 3 min in PBS/T and repeated three times. Proteins and primary and secondary antibodies were diluted in PBS/T containing 3% BSA (PBS/TB).

After blocking and washing, plates were incubated for 2 hours with doubling dilutions of wild type His-tagged HC or mutant His-tagged Hc starting from an initial concentration of 10 µg/ml protein. After washing, rabbit polyclonal anti-Hc antibody (Fairweather *et al.*, 1986) diluted 1:1000 was added for 1 hour. After washing, goat anti-rabbit antibody conjugated to horse radish peroxidase was added as a secondary antibody. Plates were devloped with o–phenylenediamine dihydrochloride (Sigma). The plates were read at 490nm in a Ceres 900 Hdi ELISA reader (Bio-Tek Instruments). Each data point represents the mean of duplicate values after subtraction of the mean value obtained when no protein was added. The GT1b binding activity of mutant proteins (Table 2) is expressed as a percentage of binding of wild-type H_C protein, and was calculated from the concentration of protein required to give an optical density at 490nm of 1.5, which represents half maximal binding of wild-type H_C protein.

Negative controls for non-specific binding included incubation of cells with all reagents except either Hc protein or primary antibody.

(ii) Competition assay

A variation of the direct binding assay was performed as follows. Coating of plates with gangliosides and blocking was as in the direct assay, and all subsequent incubations were at 37°C in 50 µl volumes. All washes were for 3 min in PBS/T and repeated three times. Proteins and secondary antibodies were diluted in PBS/TB. A constant amount of biotinylated His-tagged Hc (prepared as described above) at a concentration of 340 µg/ml in a volume of 25 µl was mixed with 25 µl of test protein at decreasing concentrations (highest concentration of 500 µg/ml). 50 µl of the mixture of biotinylated Hc/unlabelled protein was added to the ganglioside coated plates, and the plates incubated for 2 hours. The plates were washed again and incubated with streptavidin-HRP (Dako), diluted 1 in

500, for 1 hour. After washing, the plates were developed with *o*–phenylenediamine dihydrochloride (OPD, Sigma) and read at 490nm in an ELISA plate reader.

Binding of mutants to N18 cells

N18 RE-105 cells (ECACC number 88112301) were cultured in DMEM (Life Technologies) supplemented with 4 mM glutamine (Sigma), 10% FBS and 100u penicillin, 100 µg/ml streptomycin (Life Technologies). One day prior to use in indirect immunofluorescence, 3 x 10^4 cells in 0.5 ml were plated in each well of an 8 well LabTek chambered slide (Life Technologies) pretreated overnight with 5 µg/ml poly-L-lysine. Cells were fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature. All subsequent incubations were carried out at 4°C. All washes were in PBS containing 0.1% BSA (PBS/B) at 4°C and were repeated 3 times for 3 min. Cells were incubated with wild type or mutant proteins (5 µg/ml) for 2 hours. After washing, cells were incubated for 30 min in PBS/B containing 5% normal swine serum, followed by 30 min with rabbit polyclonal anti Hc antibody, diluted 1 in 500 in PBS/B containing 5% normal swine serum. The cells were washed again and incubated for 1 hour with FITC-conjugated goat anti-rabbit serum (Dako) diluted 1 in 500 in PBS/B. After washing, cells were mounted in an antifade agent in glycerol/PBS (Citifluor, Agar Scientific, Harlow, UK) and visualised by fluorescent microscopy using a Zeiss 510 confocal microscope.

Purification of rat spinal cord motoneurons

Rat spinal cord motoneurons were purified from E14 rat embryos as previously described. Briefly, ventral spinal cords were dissected, dissociated with 0.025% trypsin (GIBCO-BRL, USA) for 10 minutes at 37 °C and triturated. Cells were centrifuged through a BSA cushion and then through a metrizamide density gradient. The interphase and pellet cells were collected through a BSA cushion and resuspended in 50 µl of phosphate-buffered saline (PBS) with 0.5% BSA (PBS-BSA) plus 50 µl of mouse anti-rat p75 hybridoma supernatant, which recognises the p75 low affinity nerve growth factor (NGF) receptor specifically expressed by rat motoneurons at E14. Cells were incubated with the p75 hybridoma supernatant at 12°C for 15 minutes, washed with PBS-BSA and centrifuged through a BSA cushion. The pellet was resuspended in 80 µl of PBS-BSA and incubated with 20 µl of goat anti-mouse IgG magnetic microbeads (Miltenyi Biotec, Bergisch

Gladbach, Germany) for 15 minutes at 12°C. After another centrifugation through a BSA cushion cells were resuspended in 500 µl of PBS-BSA and purified by magnetic cell sorting using a Mini Macs magnet and a large cell separation column (Miltenyi Biotec). Purified motoneurons were seeded on glass coverslips coated with polyornithine and laminin (Sigma, UK) at a density of 40000 cells/coverslip. Cultures were maintained in a humidified 7.5% CO_2 incubator at 37°C in Neurobasal medium (Life Technologies Gibco-BRL, USA) containing B27 supplement (Gibco-BRL, USA), 2% horse serum, 0.5 mM L-glutamine, 25 µM 2-mercaptoethanol (Fluka, UK), 10 ng/ml rat ciliary neurotrophic factor and 100 pg/ml rat glial cell line-derived neurotrophic factor (both from R&D systems, USA). L-glutamate (25 µM) was added to the medium for the first four days in culture.

Binding of wild-type and mutant H_C proteins to primary motoneurones.

Cells on coverslips were cooled to 4°C for 15 minutes and then washed twice with Hanks' solution (20mM Hepes-Na, pH7.4, 0.44mM mM KH_2PO_4, 0.42mM Na_2HPO_4, 5.36mM KCl, 136mM NaCl, 0.81mM $MgSO_4$, 1.26mM $CaCl_2$, 6.1mM glucose) before incubation with wt or mutant Hc at a concentration of 5µg/ml in Hanks/0.1% BSA for 1 hour at 4 °C. After washing twice with Hanks' solution, cells were fixed in 3.7% paraformaldehyde in PBS for 15 minutes at room temperature. After washing twice with PBS, cells were incubated for 20 minutes with 50mM NH_4Cl in PBS, washed twice again with PBS and blocked for 1 hour with 2% BSA, 0.25% porcine skin gelatin, 0.2% glycine, 10% foetal calf serum. Cells were washed twice in PBS then incubated for 1 hour with rabbit anti-Hc polyclonal antiserum diluted 1 in 500 in PBS containing 1% BSA, 0.25% gelatin. After two further washes with PBS, cells were incubated for 30 min with FITC conjugated swine anti-rabbit serum (Dako) diluted 1 in 1000 in PBS. After four washes with PBS, coverslips were mounted on slides in Mowiol 4-88 (Harco), stored at 4°C and were analysed by fluorescence using a Zeiss LSM510 confocal microscope.

Retrograde transport of wild type and mutant H_C proteins

10 week old B6D2F1 mice were obtained from Harlan-Olac and were anaesthetised by intramuscular injection with Vetalar (100mg/ml) and Rompun (20 mg/ml) in a 2:1 ratio. Whilst under general anaesthesia, groups of three mice were injected in the tongue with 22 µl of PBS or PBS containing 70 - 85 µg protein using a 25µl Hamilton syringe with a 33

gauge needle. All animals recovered quickly and were left for 24 hrs to allow the injected protein to be transported to the brainstem. The mice were killed by transcardiac perfusion with PBS, followed by 4% paraformaldehyde in PBS whilst under deep anaesthesia. Brains were dissected and post-fixed for at least 1 hour in 4% formaldehyde before being transferred to 15% sucrose/PBS at 4°C for at least 16 hrs. In experiment 1 (Fig. 6, panels a-c), brains were mounted in Tissue-Tek before sectioning into 20μm slices using a cryostat. In experiment 2 (Fig. 6, panels d-h), brains were frozen in liquid nitrogen before sectioning.

Immunostaining of wt and mutant Hc in the hypoglossal nucleus

Sections were collected on slides which were precoated with poly-L-lysine (Sigma). All subsequent washes were in PBS for 5 minutes and repeated three times. After ringing with a wax pen, the sections were washed. After blocking for 30 mins with PBS/5% normal swine sera (Dako) sections were incubated with PBS/1% BSA/0.1% Triton X -100 containing rabbit anti-Hc polyclonal antibody diluted 1:500 for 2 hours. After washing, sections were incubated with biotinylated swine anti-rabbit antibody (Dako) diluted 1:500 in PBS for 2 hours. After washing again, sections were incubated with peroxidase-conjugated avidin diluted 1:500 in PBS for 2 hours. Sections were washed and developed with diaminobenzidine (0.05%) in PBS with 0.04% $NiCl_2$ and 0.01% H_2O_2 for 10 minutes. Sections were washed in distilled water, counterstained with 1% neutral red, dehydrated and mounted. Slides were stored at -50°C before photography.

Example 1 – Roles of N and C termini of H_C in ganglioside binding

An inspection of the 3-D structure of the H_C fragment TeNT (Umland *et al.*, 1997) reveals two domains, an N-terminal jelly roll domain and a C-terminal β trefoil domain, linked by a single chain (Figure 1). These domains each consist largely of β sheets joined by loops which protrude from the molecule.

Our previous study analysed neuronal cell binding of a number of malE-H_C fusion proteins. Proteins M1453 containing the entire H_C domain of TeNT (K865 – D1315) and M338 (N944 – D1315) bound avidly to primary dorsal root ganglion cells, whereas M1321 (K865

- H1271) displayed no binding in this assay. In this study, it was found that both M1453 and M338 bound equally well to GT1b gangliosides, whereas M1321 had no detectable binding. These results confirm that the first 80 amino terminal residues of H_C, absent in M338, are not essential for ganglioside or cell binding while the carboxy terminal domain, absent in M1321, appears to be essential for these activities.

Deletion mutagenesis of the C-terminal region of H_C identified V1306 – D1315 as important for ganglioside binding and neuronal cell binding. This peptide originates within the β trefoil structure, and terminates in the cleft dividing this domain and the lectin like domain (Fig. 1). To analyse further the regions of H_C involved in ganglioside GT1b binding, a series of mutant H_C proteins was constructed as His-tag fusions (Table 2). Mutant His-tagged proteins were expressed in *E.coli* BL21 and purified using nickel agarose affinity chromatography. Our first series of mutants was constructed in the C terminal region of H_C. Mutant M5, lacking the 10 C terminal residues (Δ1306-1315), displayed GT1b binding at 80% of the wild-type level, while mutant M13 (Δ1311-1315) had levels of binding indistinguishable from wild-type H_C (Table 2). To assess the role of individual amino acids within these 10 residues, several mutants containing single amino acid substitutions were constructed and GT1b ganglioside binding measured. These mutant proteins, T1308A, D1309A and E1310A all retained between 80 - 100% of the GT1b binding activity of wild-type H_C (Table 2). A number of other single amino acid substitutions within the β trefoil domain were also constructed and in all cases the proteins were found to have wild-type levels of GT1b binding activity (data not shown).

Example 2 - Mutants of H_C displaying decreased ganglioside binding

In order to identify other residues of the H_C molecule involved in ganglioside GT1b binding, we chose to investigate the regions comprising the loops joining the β sheets within the carboxy terminal β trefoil domain. These regions are surface exposed and are most likely to interact with ligands on the surface of cells. Mutants were constructed which lacked either six residues, M28 (ΔQ1274 - P1279), or twelve residues, M37 (ΔH1271 - D1282), of a loop joining two β sheets within the β trefoil domain (Fig. 1). Purified proteins were assayed for ganglioside GT1b binding. Mutant M28 retained only 5.19% of

the GT1b binding activity of wild type H_C fragment, while mutant M37 displayed an even lower level of binding, having only 1.06% of the activity of wild-type H_C fragment (Fig. 2 and Table 2). In other words, deletion of six residues resulted in 5% of wild type ganglioside binding, and deletion of the twelve residues resulted in only 1% of wild-type binding.

A second surface exposed loop was identified in the region of D1214 - N1219, connecting two β sheets within the β trefoil domain. Mutant M58 (ΔD1214 - N1219) exhibited substantially reduced ganglioside GT1b binding, at a level of 0.6 % of wild type H_C fragment (Fig. 2 and Table 2).

TABLE 2: Ganglioside binding of mutant H_C proteins

Mutant name and amino acid mutation(s) introduced	% binding relative to wild type H_C
M5 ΔV1306 - D1315	80.0 ± 15.0
T1308A	80.6 ± 2.4
D1309A	96.7 ± 5.8
E1310A	86.5 ± 19.1
M13 ΔG1311 - D1315	98.0 ± 3.5
M28 ΔQ1274 - P1279	5.19 ± 0.548
M37 ΔH1271- D1282	1.06 ± 0.587
M40 ΔQ1274 - P1279, H1293S	4.2 ± 0.72
M58 ΔD1214 - N1219	0.625 ± 0.177
M564 H1293S	42.97 ± 11.44
M57 H1293A	14.9 ± 5.37

These results were confirmed using a competition assay as described in the methods section. Thus our results demonstrate that a deletion in either one of two loops joining β sheets in the TeNT H_C molecule dramatically reduces the ability of the molecule to bind to gangliosides.

Photoaffinity labelling of TeNT with derivatised gangliosides have shown His-1293 to be involved in ganglioside binding. We tested the role of this residue in binding by mutation to both Serine and Alanine. Both these mutants showed decreased binding (to 43% and 15% of wild type respectively) confirming the importance of His 1293 in binding. His 1293 is spatially near the His-1271 to Asp 1282 loop we have now shown also to be involved in binding. A double mutant, comprising His 1293 → Ser and ΔHis 1271 to Asp 1282 was constructed and found to exhibit markedly reduced binding (4.2% of wild-type levels) (see Table 2 and Figure 2).

We also investigated the capacity of the mutant H_C proteins to bind to N18 neuronal cells (see methods).

Example 3 - Binding to primary motoneurones is retained by Histidine 1293 mutants and reduced in loop mutants.

To determine whether the ganglioside binding activity of mutant H_C proteins correlated with binding to neuronal cells, we tested the binding of the proteins to primary rat spinal cord motoneurons. Binding was visualised using a polyclonal anti-H_C antibody and fluorescent labelled secondary antibody. Both wild type H_C and mutant M5 (ΔV1306-D1315) proteins were shown to bind strongly to primary spinal cord motoneurons. These results suggest that the carboxy terminal ten residues of H_C are not essential for binding to neuronal cells. In contrast, the ability of loop mutant M58 (ΔD1214 - N1219) to bind to spinal cord motoneurons was very much reduced, as visualised by the very weak staining of primary motoneurons. Mutant M37 (ΔH1271-D1282) containing a 12 amino acid deletion in the second loop studied also showed very much reduced binding to primary spinal cord cells, as did mutant M28 (ΔQ1274-P1279) which contains a 6 amino acid deletion within the same loop region. For mutants M58, M37 and M28 the reduced binding of gangliosides GT1b (Fig. 2) is consistent with reduced binding to neuronal cells suggesting that residues within loops 1214-1219 and 1274-1279 are important for both ganglioside and cell binding.

The cell binding activities of the Histidine 1293 mutants were also studied using primary motoneurones. Both the H1293A and H1293S mutant proteins appeared to bind as strongly to primary cells as wild-type H_C protein as judged by strong staining pattern obtained. This is in contrast to the results obtained with ganglioside GT1b, where a reduction in binding activity to 12 - 43% of wild-type levels was clearly observed (Table 2). Thus a large decrease in ganglioside GT1b binding does not reduce the ability of these mutant proteins to bind to primary neuronal cells. The ganglioside binding, cell binding and retrograde transport (see below) activities of selected mutants are summarised in Table 3.

TABLE 3: Binding and transport properties of mutants of TeNT H_C in this study

The properties of selected mutant H_C proteins are summarised and compared to wild-type protein. n.d., not determined.

Protein	GT1b binding (%)	Motoneuron cell binding	Retrograde transport
wild-type H_C	100	+	+
M5 (Δ 1306-1315)	80	+	+
H1293A	12.5	+	+
H1293S	43	+	+ [a]
M28 (Δ1274-1279)	5.2	-	-
M37 (Δ1271-1282)	1.1	-	n.d.
M58 (Δ1214-1219)	0.5	-	-

Example 4 - Retrograde transport activity is retained by Histidine 1293 mutants but abolished in loop mutants.

As a functional test of the biological activity of our mutants, we investigated the ability of proteins to undergo retrograde transport in the mouse from peripheral neurons to the central nervous system. This is a well-recognised property of the H_C fragment of TeNT, and reflects the ability of TeNT to be transported to higher centres of the central nervous system. For this assay, mice were injected in the tongue with wild-type or mutant proteins

and the ability of the proteins to be transported to the hypoglossal nucleus within the brainstem was visualised. Wild-type H_C protein was detected in the hypoglossal nucleus after 24 hours, while in contrast mutant M28 (ΔQ1274 –P1279) could not be detected, indicating this mutant is defective in retrograde transport. The lack of staining of M28 was similar to that seen with the negative control, where PBS was used. This result is consistent with the reduced ganglioside binding activity of M28 (Fig. 2 and Table 2) and the reduced binding to primary motoneuron cells.

In a second experiment, we found that the loop mutant M58 (Δ1214-1219) also failed to undergo retrograde transport, the lack of staining being similar to the uninjected mouse. These results suggest that residues 1274-1279 and 1214-1219 are essential for retrograde transport, consistent with their role in ganglioside binding and cell binding. Positive staining was obtained with the C terminal mutant M5 (ΔV1306 –D1315) and wild-type H_C protein indicating that the carboxy terminal 10 residues of H_C are not essential for ganglioside binding, cell binding or retrograde transport. Finally, the role of Histidine 1293 in retrograde transport was also examined. H1293A protein underwent retrograde transport as visualised by staining of the hypoglossal nucleus, suggesting that Histidine 1293 is not absolutely required for retrograde transport activity. Similar results showing retrograde transport of another Histidine 1293 mutant (H1293S) were obtained by P. Fishman (pers. comm).

References

Fairweather *et al.*, 1986, *J. Bacteriol*, **165**, p21-27.

Eisel, et al., 1986, *Embo J.* **5(10)**: 2494-2502.

Makoff, A. J., S. P. Ballantine, A. E. Smallwood, and N. F. Fairweather, 1989, *Bio/Technology*. 7:1043-1046.

Halpern and Loftus, 1993, *J. Biol. Chem.* **268**: 11188-11192.

Shapiro *et al.*, 1997, *J. Biol. Chem* **272**: 30080-30386.

Umland *et al.*, 1997, *Nature Struct. Biol.* **4**:788-791.

Minton, N. P. 1995. Molecular genetics of clostridial neurotoxins, p. 161-194. In C. Montecucco (ed.), Clostridial neurotoxins. Springer, New York.